

## RESEARCH

# Stabilization of Quaternary Structure of Water-Soluble Quinoprotein Glucose Dehydrogenase

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## Abstract

Water-soluble quinoprotein glucose dehydrogenase (PQQGDH-B) is a dimeric enzyme whose application for glucose sensing is the focus of much attention. We attempted to increase the thermal stability of PQQGDH-B by introducing a disulfide bond at the dimer interface. The Ser residue at position 415 was selected for substitution with Cys, as structural information revealed that its side chains face each other at the dimer interface of PQQGDH-B. PQQGDH-B with Ser415Cys showed 30-fold greater thermal stability at 55°C than did the wild-type enzyme without any decrease in catalytic activity. After incubation at 70°C for 10 min, Ser415Cys retained 90% of the GDH activity of the wild-type enzyme. Disulfide bond formation between the mutant subunits was confirmed by analyses with sodium dodecylsulfate–polyacrylamide gel electrophoresis in the presence and absence of reductants. Our results indicate that the introduction of one Cys residue in each monomer of PQQGDH-B resulted in formation of a disulfide bond at the dimer interface and thus achieved a large increase in the thermal stability of the enzyme.

**Index Entries:** Pyrroloquinoline quinone (PQQ); glucose dehydrogenase (GDH); thermal stability; dimer interface; subunit–subunit interaction.

## 1. Introduction

Water-soluble quinoprotein glucose dehydrogenase (PQQGDH-B or sGDH) has great potential as a component of electron mediator-type glucose sensors (1–12). This enzyme shows 10-fold greater catalytic activity than glucose oxidase (GOD), the enzyme most widely utilized in glucose sensing. Moreover, PQQGDH-B does not use oxygen as an electron acceptor during the oxidation of glucose. These properties may eventually make PQQGDH-B an ideal glucose sensor constituent, with improved accuracy and rapidity as compared with GOD. However, PQQGDH-B lags behind GOD in terms of thermal stability, which is particularly important during the enzyme preparation and sensor fabrication processes.

We have been conducting protein engineering studies to improve the substrate specificity and thermal stability of both membrane-bound PQQGDH (PQQGDH-A) and the water-soluble form of PQQGDH (PQQGDH-B) (13–26). The

Ser231Lys mutant of PQQGDH-B had increased thermal stability at 55°C as compared with the wild-type enzyme without any decrease in catalytic activity (24). Analysis of a 3D computer model indicated that the Ser231Lys substitution may result in increased hydrophobic interactions near the mutation site, thus leading to improved thermal stability.

Assuming that the first step of heat inactivation of PQQGDH-B is dimer dissociation, the stabilization of quaternary structure would be expected to increase the enzyme's thermal stability. We previously reported that chemically cross linking the subunits of PQQGDH-B with glutaraldehyde resulted in a significant increase in the thermal stability of the enzyme (12). Glucose sensors with high operational stability were constructed with the cross-linked enzyme. We have also reported on a tethered PQQGDH with improved thermal stability, constructed by genetically fusing it to a linker peptide (25). Although a decrease in catalytic

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activity was observed in both cases, these reports indicated that stabilization of the quaternary structure improves the thermal stability of PQQGDH.

Stabilization of subunit-subunit interactions (homo- or heterooligomer) by introducing disulfide bonds at the subunit-interface has been reported for other proteins (28–30). We therefore attempted to improve the stability of PQQGDH-B by introducing a disulfide bond at the dimer interface. Analysis of the 3D structure information for PQQGDH-B (PDB code 1QBI [31–33], Fig. 1) indicates that Ser415 is located in loop 5CD and is not related to the active site. It also revealed that the side chains of Ser415 face each other at the dimer interface, and are 6.12 Å apart (O $\gamma$ –O $\gamma$ ). Ser415 was therefore selected for site-directed mutagenesis to Cys in order to introduce a disulfide bond.

## 2. Materials and Methods

### 2.1. Site-Directed Mutagenesis

The structural gene for wild-type PQQGDH-B was initially amplified by polymerase chain reaction (PCR) and inserted in the expression vectors pTrc99A (Pharmacia) and pGB (24). A 1.2-kbp *KpnI*–*HindIII* fragment containing the PQQGDH-B gene from pGB was then inserted into the pKF18k mutagenesis vector (Takara). Site-directed mutagenesis was conducted with the Mutan-Express Km kit (Takara) according to the manufacturer's instructions, with the following oligonucleotides:

5'–CATCATAAGTAGTGCAATAAGTTGG  
ATC–3' (Ser415Cys)

5'–CATCATAAGTAGTGTCATAAGTTGG  
ATC–3' (Ser415Asp)

5'–CATCATAAGTAGTGCGATAAGTTGG  
ATC–3' (Ser415Arg)

The mutations were confirmed with an automated DNA sequencer (ABI PRISM Genetic analyzer 310; Applied Biosystems). The mutated genes were digested with *KpnI* and *HindIII* and were replaced into pGB to construct expression vectors containing mutated PQQGDH-B. Numbering of the amino acid positions starts from the first residue of the signal peptide (24 residues).

### 2.2. Enzyme Preparation and Assay

PQQGDH-B enzyme was prepared as previously reported (24,26). GDH activity was measured by using 0.6 mM phenazine methosulfate and 0.06 mM 2,6-dichlorophenolindophenol, following a 30-min preincubation in 10 mM 3-(*N*-morpholino)propane sulfonate (MOPS)–NaOH (pH 7.0) containing 1  $\mu$ M pyrroloquinoline quinone (PQQ) and 1 mM CaCl<sub>2</sub> at room temperature (25°C). The enzyme activity was determined by measuring the decrease in absorbance of dichlorophenol-indolphenol (DCIP) at 600 nm. The substrate specificity profiles were determined by using the following 6 substrates: glucose, allose, 3-*O*-methylglucose, galactose, lactose, and maltose.

### 2.3. Analysis of Stability of PQQGDH-B

The thermal stability of wild-type and mutant PQQGDH-B was determined with 0.3  $\mu$ g/mL protein, as previously reported (24). Since the initial time course for thermal inactivation at 55°C followed first-order kinetics, the thermal stability of each mutant enzyme was expressed as a half-life. Following the 30-min preincubation described above, each enzyme sample was subjected to thermal inactivation experiments. Thermal inactivation was measured by incubating the holoenzyme in a total volume of 200  $\mu$ L of 10 mM MOPS–NaOH (pH 7.0) at 55°C. Aliquots were taken every 5 min and kept at 4°C for 2 min, followed by incubation at room temperature for 30 min. The residual enzyme activity was determined as described above. The thermal stability of Ser415Cys was also determined by incubating purified enzyme at various temperatures for 10 min. The residual activities were determined as described above, and were compared with the initial activity.

Tolerance to ethylenediamine tetraacetic acid (EDTA) was determined by incubating wild-type and mutant PQQGDH-B in 10 mM MOPS–NaOH (pH 7.0) containing 5 mM EDTA and periodically measuring the residual activity of aliquots.

### 2.4. Prediction of Three-Dimensional Structure

Prediction of three-dimensional structure was done with the Molecular Operating Environment (MOE) (Chemical Computing Group).

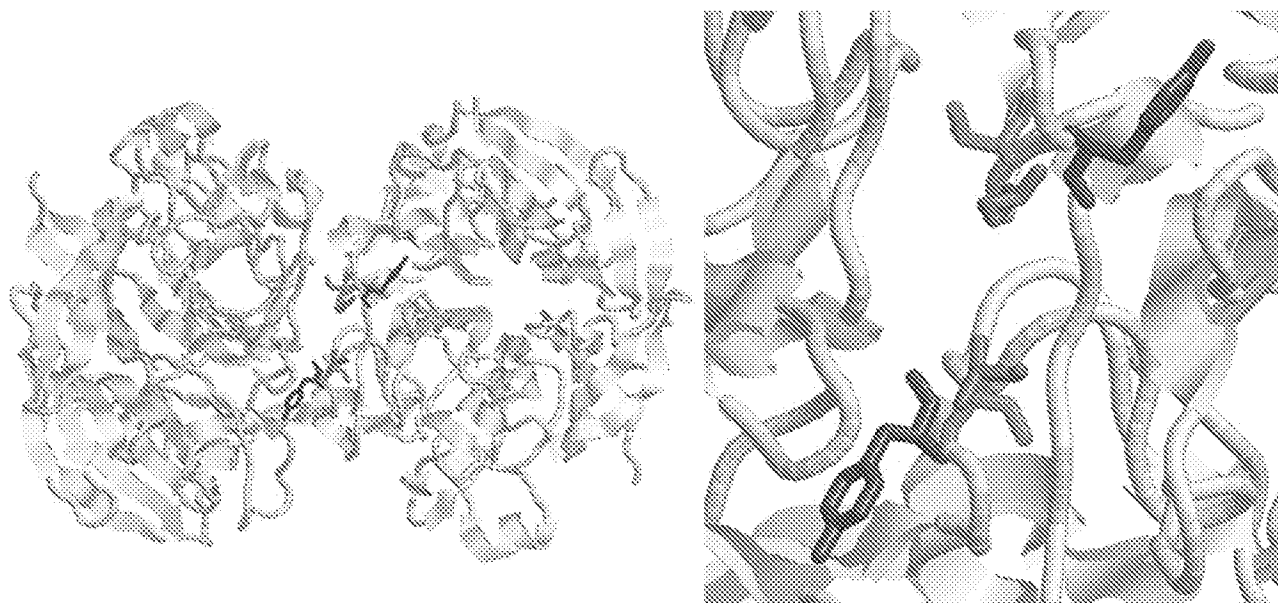


Fig. 1. Structure of PQQGDH-B (PDB code; 1QBI [31]) and dimer interface. Tyr414, Ser415 and Thr416 are displayed in stick representation.

## 2.5. Disulfide-Bond Detection Methods

Purified enzyme samples were solubilized at room temperature or by heating (15 min, 60°C) in sodium dodecylsulfate (SDS)-containing sample buffer in the absence or presence of 2-mercaptoethanol (5%, v/v). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using Phastgel Gradient 8–25 gels on a Phastsystem (Amersham Biosciences). After electrophoresis, the proteins were detected by silver staining.

## 3. Results

### 3.1. Thermal Stability of Ser415 Variants

We constructed three variants of PQQGDH-B by substituting Ser415 with Cys, Asp, and Arg through site-directed mutagenesis. According to the thermal stability data at 55°C, summarized in Fig. 2, only Ser415Cys showed a drastic increase in thermal stability over the wild-type enzyme. Substitution of Ser415 with either Asp or Arg resulted in a slight decrease in thermal stability. The Ser415Cys mutant was then purified through cation exchange chromatography to investigate the enzyme's properties in greater detail.

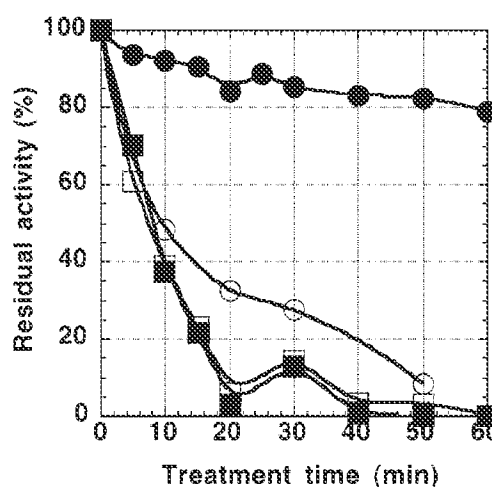


Fig. 2. Thermal stability of Ser415 variants of PQQGDH-B. Aliquots were taken every 5 min to measure residual activity from the following protein samples (0.3 µg/mL) incubating at 55°C: Wild-type, ○; Ser415Cys, ●; Ser415Asp, □; Ser415Arg, ■.

We also compared results of Ser415Cys with those of Ser231Lys, which showed an eight-fold greater thermal stability than the wild-type enzyme (Fig. 3A). The thermal inactivation of Ser415Cys, Ser231Lys, and wild-type PQQGDH-B followed first-order kinetics. From linear regres-

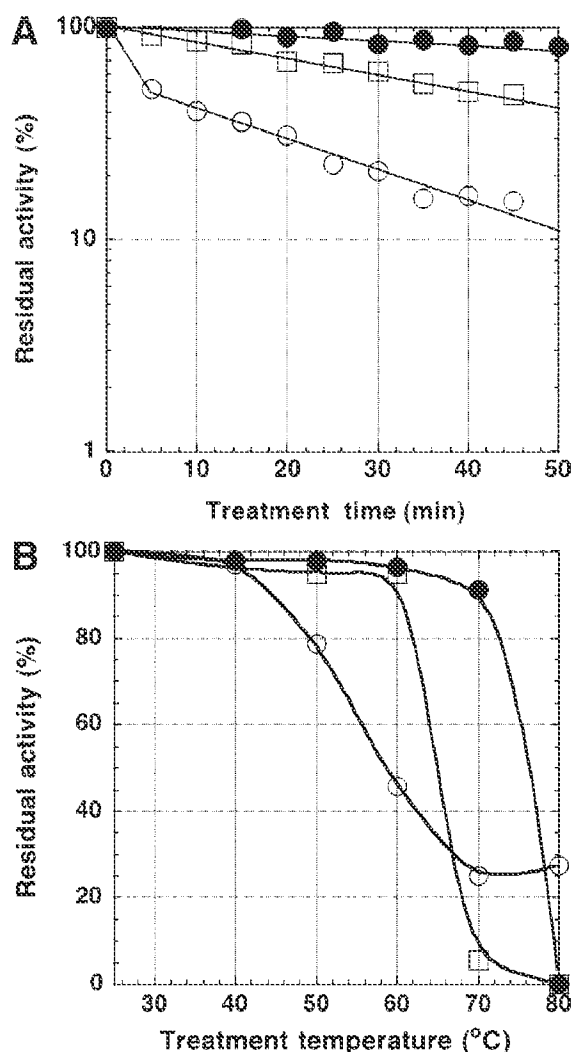


Fig. 3. (A) Thermal stability of PQQGDH-B enzymes at 55°C. Aliquots were taken every 5 min to measure residual activity from the following protein samples (0.3  $\mu\text{g/mL}$ ) incubating at 55°C: Wild-type,  $\circ$ ; Ser415Cys,  $\bullet$ ; Ser231Lys,  $\square$ . (B) Thermal stability of PQQGDH-B enzymes at indicated temperatures. Residual activity was measured at 25°C after 10 min of incubation of the following protein samples (0.3  $\mu\text{g/mL}$ ) at different temperatures: Wild-type,  $\circ$ ; Ser415Cys,  $\bullet$ ; Ser231Lys,  $\square$ .

sion of the logarithmic curves of residual activity against time, we evaluated the thermal stabilities of the variants and wild-type enzyme at 55°C and expressed the results as half-life times. The half-life at 55°C of Ser415Cys (183 min) was approx 36-fold greater than that of the wild-type enzyme

(5 min) and 4-fold greater than that of the Ser231Lys variant (40 min).

By conducting 10-min incubations at different temperatures, we found that inactivation of wild-type PQQGDH-B started at approx 50°C, while Ser231Lys and Ser415Cys showed much greater thermal stabilities. With 60°C treatment, Ser231Lys and Ser415Cys showed no significant inactivation. Ser231Lys was inactivated by heating at 70°C for 10 min, while Ser415Cys needed treatment at 80°C to cause a comparable inactivation. These results indicate that the replacement of Ser415 with Cys achieved a dramatic stabilization of PQQGDH-B.

### 3.2. Kinetic Parameters of Ser415Cys

The kinetic parameters of Ser415Cys with various substrates are summarized in Table 1. The substitution of Ser415 with Cys did not result in a decrease in GDH activity (3461  $\text{s}^{-1}$  at 25°C). The  $K_m$  value of Ser415Cys for glucose is 16 mM, which is lower than that of the wild-type enzyme (27 mM). The substitution also caused a decrease in the  $K_m$  values for other substrates, such as all-ose (from 36 mM to 21 mM) and maltose (from 26 mM to 13 mM). In contrast,  $k_{cat}$  values were increased or were similar to those of the wild-type enzyme. The calculated catalytic efficiency ( $k_{cat}/K_m$ ) was therefore improved over that of the wild-type enzyme. These results indicate that the Ser415Cys substitution did not have any significant detrimental effect on the kinetic parameters of PQQGDH-B.

### 3.3. Disulfide Bond Formation with SDS-PAGE Analysis

In order to investigate the impact of the Ser415-to-Cys substitution on the quaternary structure of PQQGDH-B, we first subjected this mutant to SDS-PAGE in the presence and absence of the reductant 2-mercaptoethanol (2-ME) (Fig. 4). SDS-PAGE analyses in the presence of 2-ME showed that both wild-type PQQGDH-B and the Ser415Cys variant migrated as a single band corresponding to a molecular weight of 50-kDa, indicating that each enzyme was composed of identical subunits. On the other hand, in the absence of

Table 1  
Kinetic parameters of Wild-Type and Ser415Cys PQQGDH-B for Various Substrates

	Wild-type			Ser415Cys		
	<i>K<sub>m</sub></i> (mM)	<i>k<sub>cat</sub></i> (s <sup>-1</sup> )	<i>k<sub>cat</sub>/K<sub>m</sub></i> (s <sup>-1</sup> mM <sup>-1</sup> )	<i>K<sub>m</sub></i> (mM)	<i>k<sub>cat</sub></i> (s <sup>-1</sup> )	<i>k<sub>cat</sub>/K<sub>m</sub></i> (s <sup>-1</sup> mM <sup>-1</sup> )
Glucose	27	3436	127 (100%)	16	3461	216 (100%)
Allose	36	2509	70 (55%)	21	3080	147 (68%)
3- <i>O</i> -m-glucose	29	3011	104 (82%)	30	4890	163 (75%)
Galactose	5	232	46 (36%)	7	283	40 (19%)
Lactose	19	1659	87 (69%)	19	2591	136 (63%)
Maltose	26	1930	74 (58%)	13	2093	161 (75%)

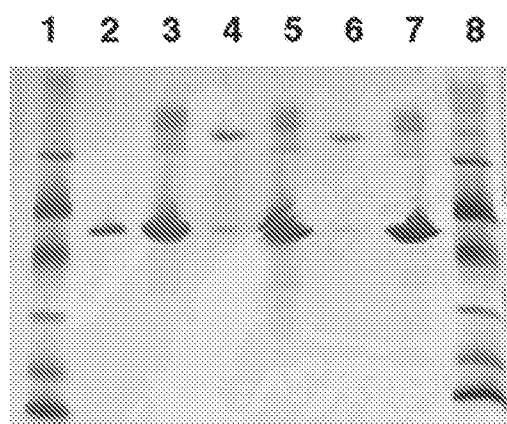


Fig. 4. SDS-PAGE analysis of wild-type and Ser415Cys PQQGDH-B enzymes. Lanes 1, 8: Low-molecular-weight marker (Amersham Biosciences), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and  $\alpha$ -lactalbumin (14.4 kDa). Lane 2: 2-ME-treated Ser415Cys. Lane 3: 2-ME-treated wild-type enzyme. Lanes 4, 6: Ser415Cys without 2-ME treatment at 60°C and 25°C, respectively. Lanes 5, 7: Wild-type enzyme without 2-ME treatment at 60°C and 25°C, respectively.

2-ME, Ser415Cys migrated as 100-kDa band, which corresponds to the molecular weight of a PQQGDH-B dimer. In contrast, the wild-type protein continued to migrate as a 50-kDa band in the presence of reducing agent. The wild-type sample was intentionally overloaded to confirm the absence of the dimer form. This resulted in the appearance of larger-sized proteins, none of which corresponded to 100-kDa-sized proteins. Furthermore, these larger proteins appeared in both the presence and absence of reducing agents. These

results confirm that Ser415Cys forms a homodimeric enzyme covalently joined by a disulfide bond at the dimer interface of PQQGDH-B.

#### 4. Discussion

We constructed a mutant enzyme with improved thermal stability by substituting an amino acid residue located at the dimer interface of PQQGDH-B. In the PQQGDH-B dimer, the side chains of the selected residue (Ser415) face each other at the dimer interface. We therefore expected that replacement of Ser415 by Cys would result in the formation of a disulfide bond between each monomer, leading to stabilization of the quaternary structure of PQQGDH-B. The resulting mutant enzyme, Ser415Cys, is the most stable PQQGDH variant so far reported.

The challenges for stabilization of quaternary structure by introducing disulfide bonds have been previously reported for other proteins (28–30). The introduction of an additional disulfide bond is often a more effective method for the stabilization than is the engineering of other interactions, such as hydrophobic interactions and hydrogen bonds. However, because of the rigid covalent bonds they may contain, such engineered enzymes often lose their conformational flexibility, leading to decreased activity (operator DNA binding affinity [28], receptor binding activity [29]) and stability (30). In proteins with quaternary structure, the active site is often constructed at the subunit–subunit interface or by the cooperation of each subunit. Moreover, disulfide bonds have usually been introduced by substituting more than two amino acid residues per monomer. These

mutants generally have multivalent disulfide-bond formations resulting in decreased flexibility of the quaternary structure.

On the basis of these considerations, we used 3D information to select a residue that is located at the dimer interface away from the active site of PQQGDH. Ser415 is in a unique position, with its side chain facing the side chain of Ser415 of the other monomeric molecule in the PQQGDH homodimer. By introducing a disulfide bond through the substitution of a single residue, we strengthened the enzyme's quaternary structure while maintaining its flexibility. A drastic increase in thermal stability was achieved by substituting Ser415 with Cys, without decreasing the catalytic activity of PQQGDH-B.

Such a high degree of thermal stability may facilitate the preparation of enzymes and sensors with little loss of enzyme activity. Our achievement may also extend the use of PQQGDH-B to continuous glucose monitoring (CGM) systems, currently the focus of great attention. Several studies and commercial products involving CGM systems have been reported, however, all of them employ GOD. No PQQGDH-based CGM system has yet been reported; mainly because of the low stability of PQQGDH during continuous operation at 37°C. CGM systems, either attached on the skin surface or inserted subcutaneously, must operate continuously for at least 72 h. The thermal stability of Ser415Cys is greater than that of GOD, thus eliminating an important barrier to its use in CGM systems. Ser415Cys can therefore be used in CGM systems, potentially leading to the development of a more accurate and durable glucose-sensor system.

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